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Preparation and characterization of prototypes for multi-modal separation media aimed for capture of negatively charged biomolecules at high salt conditions☆

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Abstract

Several prototypes of multi-modal ligands suitable for the capture of negatively charged proteins from high conductivity (28 mS/cm) mobile phases were coupled to Sepharose 6 Fast Flow. These new prototypes of multi-modal anion-exchangers were found by screening a diverse library of multi-modal ligands and selecting anion-exchangers resulting in elution of test proteins at high ionic strength. Candidates were then tested with respect to breakthrough capacity of BSA in a buffer adjusted to a high conductivity (20 mM Piperazine and 0.25 M NaCl, pH 6.0). The recovery of BSA was also tested with a salt step (from 0.25 to 2.0 M NaCl using 20 mM Piperazine as buffer, pH 6.0) or with a pH-step to pH 4.0. We have found that non-aromatic multi-modal anion-exchange ligands based on primary or secondary amines (or both) are optimal for the capture of proteins at high salt conditions. Furthermore, these new multi-modal anion-exchange ligands have been designed to take advantage not only of electrostatic but also hydrogen bond interactions. This has been accomplished through modification of the ligands by the introduction of hydroxyl groups in the proximity of the ionic group. Experimental evidence on the importance of the relative position of the hydroxyl groups on the ligand in order to improve the breakthrough capacity of BSA has been found. Compared to strong anion-exchangers such as Q Sepharose™ Fast Flow the new multi-modal weak anion-exchangers have breakthrough capacities of BSA at mobile phases of 28 mS/cm and pH 6.0 that are 20–30 times higher. The new multi-modal anion-exchangers can also be used at normal anion-exchange conditions and with either a salt step or a pH-step to acidic pH can accomplish the elution of proteins. In addition, the functional performance of the new anion-exchangers was found to be intact after treatment in 1.0 M sodium hydroxide solution for 1 week. A number of multi-modal anion-exchange ligands based on aromatic amines exhibiting high breakthrough capacity of BSA have been found. With these ligands recovery was often found to be low due to strong non-electrostatic interactions. However, for phenol derived anion-exchange media the recovery can be improved by desorption at high pH.
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Keywords: Multi-modal separation media; High salt conditions; Capture; Anion-exchange ligands; Library of chromatographic media

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1. Introduction

The great advances in molecular biology over the past two decades have resulted in a new era especially in large-scale production of biotherapeutics and diagnostics. The capture step in downstream purification of recombinant proteins from cell culture supernatants of a variety of genetically engineered cells serves the dual purpose of removing the bulk of the impurities and in concentrating the product of interest. Because of this, ion-exchange chromatography has become a very important tool in downstream purification for the isolation of proteins [1–5]. However, the use of classical ion-exchangers is not always optimal, as it requires dilution of the broth, due to high conductivity of the solution, to achieve an acceptable sample capacity of the ion-exchanger [6]. Dilution increases the volume of the feedstock and may not be realistic at manufacturing scale. Furthermore, the salt concentration of a cell culture broth is often too low to be applied onto a hydrophobic interaction adsorbent. Direct loading of a cell culture supernatant should substantially improve and simplify purification processes of recombinant biomolecules.

In this paper, the development, preparation and chromatographic properties of new multi-modal anion-exchangers able to adsorb proteins at high ionic strengths are presented. The different multi-modal anion-exchange media tested were obtained by coupling multi-modal amine ligands to epoxy-activated Sepharose 6 Fast Flow medium. These multi-modal ligands were chosen to generate a library of anion-exchangers with a diversity of secondary interactions such as hydrogen bonding and hydrophobic interactions. The different multi-modal anion-exchange ligands were mainly varied according to the number and type of proton donors, the position of the proton donors in relation to the anion-exchange group, and the type (weak or strong amine ligands) and number of anion-exchange groups. Both non-aromatic and aromatic ligands have also been studied. A way of finding the new kind of "high salt" anion-exchangers has been to screen for anion-exchangers resulting in high elution ionic strength for three test proteins as compared to the commercially available anion-exchanger Q Sepharose™ Fast Flow. High salt candidates (coupled ligands resulting in elution of test proteins at high conductivity) were then tested with respect to breakthrough capacity of BSA at high salt conditions (28 mS/cm) and the recovery of adsorbed BSA. For the better prototypes the functional stability after treatment for 1 week in 1 M sodium hydroxide solution was evaluated.

2. Experimental

2.1. Media and reagents

The strong anion-exchange media Q Sepharose™ Fast Flow and Q Sepharose XL and the base matrix Sepharose 6 Fast Flow, used for the chromatographic evaluation, were obtained from Amersham Biosciense (Uppsala, Sweden). All ligands used for synthesis of anion-exchange media were of p.a. quality and most of them were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Synthesis

The library of multi-modal anion-exchangers is essentially based on the coupling of a series of amine containing ligands to an activated Sepharose 6 Fast Flow. The activation is realised by derivatization of the solid support with allyl glycidyl ether followed by bromination of the allylated gel. A high degree of allylation (0.4 mmol/ml gel) was chosen for the starting matrix in order to obtain gels with different degrees of substitution. Note that due to the intrinsic differences in reactivity and nucleophilicity of the ligands it has not always been possible to obtain media with exactly the same degree of ligand substitution for direct comparison. To a certain extent low reactivity was compensated with longer reaction times and larger excesses of ligands. Thus, while in a regular protocol the ligand was normally introduced as a five-fold excess the nucleophilic substitution was carried out with a 15-fold excess in the case of a highly substituted TRIS Sepharose 6 Fast Flow.

By these methods a library of about 60 new multi-modal anion-exchange media was established. The non-aromatic amine ligands tested are presented in Table 1, and the characteristics of the aromatic ligands are reported in Table 5. Note that by coupling these amines to an activated allylated Sepharose 6 Fast Flow, the amine group will be substituted with

Table 1
Ligands used in the synthesis of different non-aromatic anion-exchangers

ILC	Ligand name	ILC	Ligand name
1	1,3-Diamino-2-propanol	25	2-Amino-1,3-propandiol
2	tris(2-Aminoethyl) amine	26	Ethanolamine
3	1-Amino-4-guanidobutane	27	Ammonia
4	1,2-Diaminoethane	28	1,3-Diaminopropane
5	1,3-Diamino-2-propanol	29	bis(TRIS[a]) pentane
6	1,2-Diaminoethane	30	Trimethylamine[b]
7	bis(3-Aminopropyl) amine	31	4-Amino-4-(3-hydoxypropyl)-1,7-heptandiol
8	1,3-Diaminopropane	32	2-Amino-2-methyl-1,3-propanediol
9	1,2-Diaminoethane	33	Diethanolamine
10	tris(Hydroxymethyl) aminomethane	34	*N*-(3-Aminopropyl) diethanolamine
11	Ethanolamine	35	*N*-Butylamine
12	1,3-Diaminopentane	36	2-(2-Aminoethoxy) ethanol
13	Polyethylenimine (MW: 2000)	37	1-Amino-1-deoxy-D-sorbitol
14	tris(Hydroxymethyl) aminomethane	38	*N,N'*-bis(2-hydroxyethyl) ethylendiamine
15	Pentaethylenhexamine	39	Triethanolamine
16	1,3-Diamino-2,2-dimethylpropane	40	3-Methylamino-1,2-propandiol
17	2-Amino-ethanethiol	41	Diallylamine
18	Diethylenetriamine	42	*N*-Methyldiethanolamine
19	1,5-Diaminopentane	43	4-Amino-4-(3-hydoxypropyl)-1,7-heptandiol
20	1,4-Diaminobutane	44	Trimethylamine[c]
21	Diethyltriamine	45	6-Amino-1-hexanol
22	tris(Hydroxymethyl) aminomethane	46	2-(Methylamino) ethanol
23	Methioninol	47	4-Amino-1-butanol
24	Hydrazine		

For some of the different immobilised ligand codes (ILC) the same ligand is used but the ligand density is different (see Cl^- capacity in Table 2).

[a] TRIS: tris(hydroxymethyl) aminomethane.

[b] Q Sepharose XL.

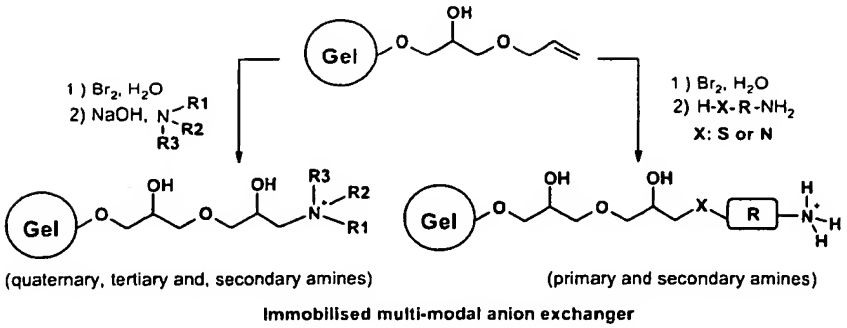
[c] Q Sepharose™ Fast Flow.

an alkyl group [7]. This means, for example, that a primary amine will be converted to a secondary after immobilisation on Sepharose 6 Fast Flow. It is also worth noticing that a polyamine will generate media with both primary and secondary amines. Thiol containing ligands such as 2-amino-ethanethiol will presumably couple mainly via the sulphur atom and give a medium with only primary amine functions (Scheme 1).

The coupling of the anion-exchange ligand was started by adding bromine (until a persistent yellow colour was obtained) to a stirred suspension of 100 ml of allyl activated Sepharose 6 Fast Flow (0.4 mmol allyl groups/ml drained gel), containing by 4 g of sodium acetate and 100 ml of distilled water. Sodium formiate was then added until the suspension was fully decolourized. The reaction mixture was filtered and the gel washed with 500 ml of distilled water. A suitable aliquot (ca. 5 ml) of the activated gel was then transferred to a reaction vessel and coupled with the appropriate multi-modal anion-exchange ligand (ca. five times excess compared to the molar amount of activated allyl groups) at pH 11.5. The reaction was stirred for 18 h at 60 °C. The suspension was filtered and the gel was successively washed with 3 ml × 10 ml of distilled water, 3 ml × 10 ml ethanol, 3 ml × 10 ml aqueous 0.5 M HCl and finally with 3 ml × 10 ml of distilled water. The degree of substitution was estimated by potentiometric titration with silver nitrate after the anion-exchange ligand was converted to its Cl^- form.

2.3. Function test

All chromatographic experiments were carried out using Amersham Biosciences ÄKTA™ explorer 10 XT system. The UV monitor (path length: 10 mm) was



Scheme 1. Synthesis of a library of multi-modal anion-exchanger media.

adjusted to 280 nm. All sample solutions and buffers were carefully filtered through a 0.45 μm Millipore millex HA filter before use.

The multi-modal anion-exchangers were packed in 1.0 ml HR 5/5 columns and equilibrated with 20 column volumes of the A-buffer (20 mM phosphate buffer; pH 6.8). Fifty microlitres of a protein mixture (6 mg/ml Conalbumin, 4 mg/ml Lactalbumin and 6 mg/ml Soybean trypsin inhibitor) was applied to the column and eluted with a linear gradient (gradient volume = 20 column volumes) to 100% of the B-buffer (A-buffer plus 2.0 M NaCl). The flow rate was adjusted to 0.3 ml/min (100 cm/h).

2.4. Breakthrough capacity ($Q_{b10\%}$) at high salt conditions

The media to be investigated were packed in HR 5/5 columns and the sample solution was BSA (4 mg/ml) dissolved in 20 mM Piperazine buffer (pH 6.0) with 0.25 M NaCl added. This solution was pumped at a flow rate of 1.0 ml/min through the column after equilibration with buffer solution. The breakthrough capacity was evaluated at 10% of the maximum UV detector signal (280 nm). The maximum UV signal was estimated by pumping the test solution directly into the detector. The breakthrough capacity at 10% of absorbance maximum ($Q_{b10\%}$) was calculated according to the formula:

$$Q_{b10\%} = (T_{R10\%} - T_{RD})\frac{C}{V_c}$$

where $T_{R10\%}$ is the retention time (min) at 10% of absorbance maximum, T_{RD} the void volume time in the system (min), C the concentration of BSA (4 mg/ml) and V_c the column volume (ml).

To investigate if the new multi-modal anion-exchange ligands also work properly at low salt conditions the breakthrough capacity was investigated at such conditions (20 mM Piperazine, pH 6.0).

2.5. Recovery

To a column (HR 5/5) equilibrated with Piperazine buffer (20 mM Piperazine, pH 6.0, with 0.25 M NaCl) was applied a solution of BSA (4 mg/ml) from a 50 ml super loop until an amount of BSA corresponding to 30% of its breakthrough capacity was applied. The column was then washed with two bed volumes of the equilibration buffer and the bound BSA was eluted with a Piperazine buffer (20 mM Piperazine, pH 6.0, with 2.0 M NaCl) or 100 mM acetate buffer adjusted to pH 4.0. In addition, adsorbed BSA on ligands (Octopamine) and (Tyrosinol) were eluted with a TRIS buffer (0.2 M TRIS, pH 9.0, with 2 M NaCl). The column effluent at elution was collected (20 ml) and the recovery was determined spectrophotometrically at 280 nm.

3. Results and discussion

Anion-exchange chromatography is primarily based on the interactions between negatively charged amino acids on the protein surface and positively charged ligands [8]. However, other interactions such as hydrophobic and hydrogen bonding may be involved. Ion-exchange media exhibiting mixed mode interac-

tions are becoming increasingly important in liquid chromatographic separations in order to optimize the selectivity [9–12]. By coupling two dissimilar ligands [13] to the base matrix or attach a multi-modal ligand [14] possessing groups that can interact with the sample in different ways; mixed mode interactions can be obtained. This work has focused on multi-modal ligands based on amines to obtain anion-exchangers able to capture proteins at high salt conditions.

3.1. Characterization of mixed-mode non-aromatic anion-exchanger aimed for capture of proteins at high salt conditions

A series of multi-modal anion-exchangers were prepared by coupling different amines to epoxide-activated Sepharose 6 Fast Flow. The non-aromatic amine ligands tested are presented in Table 1 and the way of finding "high salt" anion-exchange ligand candidates has been to screen for anion-exchangers exhibiting high elution conductivity for the three test proteins. The elution conductivity of the three test proteins and the breakthrough capacity of BSA for all ligands are summarised in Table 2. Table 2 shows that all proteins could be eluted from all tested non-aromatic anion-exchangers by a salt gradient and that much higher breakthrough capacity of BSA is obtained for a number of different ligands compared to Q Sepharose™ Fast Flow (ILC 44). It can be noted that the conductivity of the adsorption buffer used in the breakthrough capacity experiments was 28 mS/cm, in the upper range of that normally (10–30 mS/cm) used for cell culture media [15]. This means that at least 15 multi-modal non-aromatic anion-exchangers have been found which exhibit reasonably good breakthrough capacity (>15 mg BSA/ml) at high salt conditions. As expected, it is a general trend that the breakthrough capacity of BSA at high salt condition increases when the elution conductivity of the three proteins (Conalbumin, Lactalbumin and Soybean trypsin inhibitor) increases. However, there are some exceptions to this trend as there are anion-exchange ligands having the same elution conductivity for some of the proteins but with great difference in breakthrough capacity for BSA. For example, the multi-modal anion-exchangers based on ligand 12 (1,3-diaminopentane) and ligand 28 (1,3-diaminopropane) resulted in an elution conductivity of Conalbumin of 25.3 and 25.2 mS/cm, respectively. Nevertheless, the breakthrough capacity of BSA for the anion-exchanger based on ligand 12 was four times higher compared to ligand 28 (Table 2). This may indicate that the hydrophobicity of the ligands strongly affects the selectivity of BSA. To further evaluate the data in Table 2 principal component analysis (PCA) was used [16]. Any data table built up of x columns (variables) and y roads (objects) can be represented by x vector in a y-dimensional space. In multivariate analysis interpretation of the information in the data set is made by projections of the multivariate space onto planes (score plots). The planes that retain most of the available information can be determined by principal component analysis. This means that the labels of the axes in the score plot presented in Fig. 1 have no physical or chemical meaning. The score plots in Fig. 1 are used to interpret differences and similarities among different ligands. The closer the ligands are in a score plot, the more similar they are with respect to the two principal components. In this interpretation the type of ligands were considered the objects and the combinations of the elution conductivity of the three proteins and the breakthrough capacity considered the variables. According to Fig. 1A the score plot shows that the ligands (anion-exchangers based on different ligands) are distributed in all four quadrants and that two principal components explain 95% of the total variation. To simplify the interpretation of the score plot, the ligands have been coded to reflect their Q_b-values (Fig. 1B). This diagram clearly shows that all good ligands (ligands with high Q_b-values) are situated in the first and fourth quadrants and those ligands with results showing low Q_b-values are placed in the second and third quadrants. Furthermore, in Fig. 1C the ligands have been coded according to type of amine. Fig. 1C illustrates that all quaternary and tertiary amine ligands are situated in the second and third quadrants and consequently all these ligand structures are poor anion-exchange ligands at high salt conditions. Therefore, it can be concluded, that primary and secondary amines or ligands comprising both primary and secondary amines are to be preferred as high salt anion-exchange ligands. In addition, the ligands 13 (polyethylenimine) and 15 (pentaethylenhexamine) resulted in anion-exchangers with high breakthrough capacities. These results indicate that the concentra-

Table 2
Elution conductivity at pH 6.8 of three proteins and breakthrough capacity at pH 6.0 at high salt conditions of BSA on different immobilised non-aromatic anion-exchange ligands

Immobilised ligand code	Cl^- capacity (mmol/ml)	Elution conductivity (mS/cm)			$Q_{b10\%}$ of BSA (mg/ml)
		Conalbumin	Lactalbumin	STI	
1	0.50	33.8	55.1	81.5	32.3
2	0.60	38.3	60.9	89.3	28.7
3	0.29	57.3	74.2	113.2	27.7
4	0.54	23.7	45.9	70.7	26.4
5	0.30	30.1	52.1	80.1	26.0
6	0.44	22.2	42.5	68.5	25.6
7	0.70	29.4	52.5	74.5	23.1
8	0.41	29.3	51.4	72.9	22.5
9	0.46	21.4	41.2	59.7	20.4
10	0.21	34.8	41.3	60.7	20.3
11	0.36	18.5	37.5	50.8	18.9
12	0.41	25.3	55.0	69.8	18.6
13	0.45	22.3	45.8	68.8	18.4
14	0.17	27.6	37.5	54.7	17.7
15	0.53	21.3	42.5	61.8	16.7
16	0.40	20.7	48.1	65.0	14.8
17	0.29	26.9	47.3	67.8	13.9
18	0.63	26.5	48.2	67.2	12.6
19	0.41	22.1	44.2	60.1	11.4
20	0.44	24.7	47.3	65.1	11.4
21	0.33	22.2	42.8	60.5	9.8
22	0.13	24.1	31.6	49.9	8.6
23	0.22	19.6	41.8	59.3	7.8
24	0.30	9.1	18.9	27.5	6.9
25	0.14	14.2	29.6	42.3	5.7
26	0.21	23.9	36.8	50.9	5.0
27	0.15	20.2	36.6	50.3	4.6
28	0.15	25.2	39.1	58.7	4.1
29	0.09	20.8	26.4	44.2	4.0
30	0.20	5.9	27.9	30.7	3.7
31	0.07	11.7	20.2	28.5	3.6
32	0.20	26.3	39.0	53.8	3.5
33	0.22	16.8	25.5	36.1	3.2
34	0.21	19.7	31.4	44.0	3.1
35	0.19	15.0	47.0	53.2	3.0
36	0.13	11.2	18.4	26.5	2.7
37	0.09	10.6	10.6	19.9	2.7
38	0.15	17.9	28.3	38.7	2.6
39	0.14	22.0	31.6	43.6	2.5
40	0.21	11.0	27.3	36.8	2.4
41	0.22	8.0	16.0	45.9	1.5
42	0.22	19.0	29.3	40.5	1.4
43	0.17	7.8	23.5	31.3	1.1
44	0.24	12.2	20.3	29.5	1.1
45	0.16	8.0	29.4	37.4	nd[a]
46	0.21	10.1	27.0	36.3	nd
47	0.16	9.0	27.0	35.0	nd

[a] nd: Not determined.

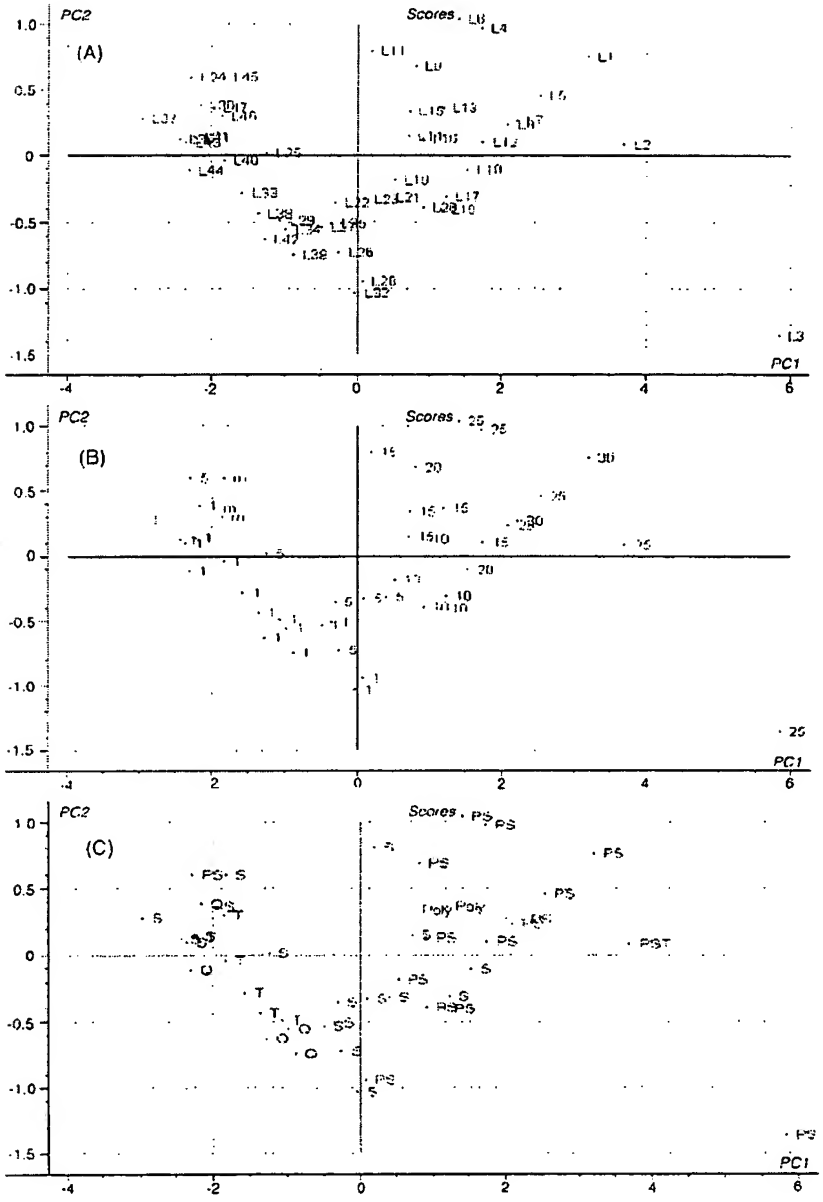


Fig. 1. Score plot (PC1 and PC2 are the scores of the first and the second principal component, respectively) of the data in Table 2. The objects are coded as ligands according to Table 1 in (A), breakthrough capacity of BSA expressed as mg/ml in (B) and type of amine group in (C). In (B) the code m, 1, 5, 10, 15, 20, 25 and 30 means a breakthrough capacity of: not determined, 1–5, 5–10, 10–15, 15–20, 20–25, 25–30, 30–35 mg/ml, respectively. In (C) the different amine groups are coded as: Q: quaternary amine, T: tertiary amines, S: secondary amines, PS: the ligand contains both a primary and a secondary amine, PST: the ligand is a primary, secondary and a tertiary amine and Poly: polyethylenimine or pentaethylenhexamine. The two principal components describe 95% of the total variation.

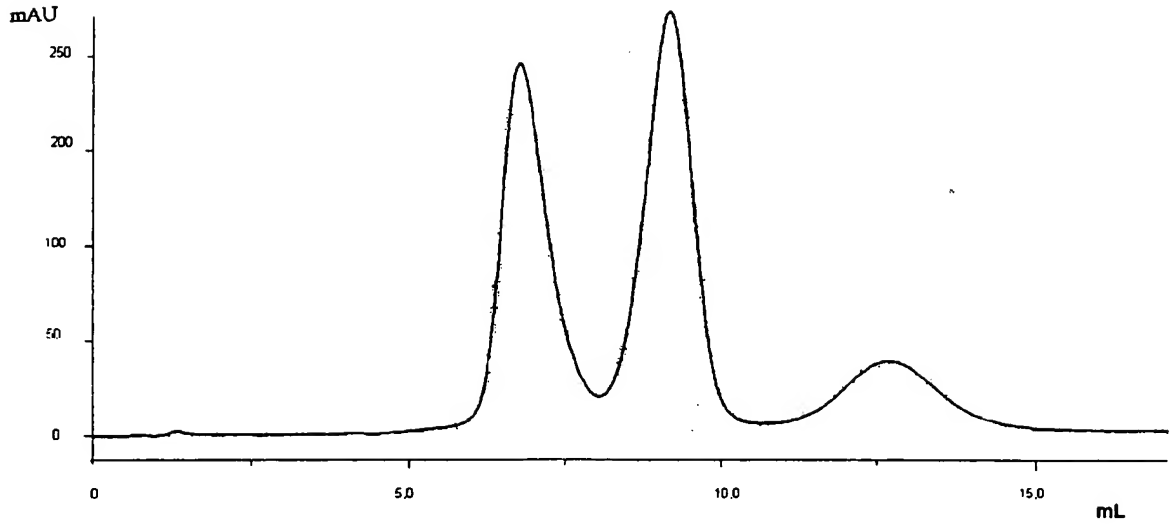


Fig. 2. A chromatogram of a protein mixture (Conalbumin, Lactalbumin and Soybean trypsin inhibitor) on an anion-exchanger based on 1,3-diaminoethane (ILC 6) and packed in an HR 5/5 column. Adsorption buffer: 20 mM phosphate buffer (pH 6.8); B-buffer: A-buffer + 2.0 M NaCl; elution conditions: linear gradient from the A-buffer to 100% B-buffer (gradient volume = 20 column volumes); flow rate: 0.3 ml/mm; injection volume 50 μl; protein concentration: 4–6 mg/ml of each protein. Conalbumin, Lactalbumin and Soybean trypsin inhibitor are eluted as the first second and third peak, respectively.

tion of counter ions in the diffuse double layer of quaternary and tertiary amine ligands is higher compared to secondary and primary amine ligands. Association of the proteins to the anion-exchanger corresponds to the expulsion of its counter ions, therefore the binding of proteins at high salt conditions to primary or secondary amine ligands will be more energetically favourable. Also, primary and secondary amines have more hydrogen atoms that can participate in hydrogen bonding as compared to tertiary and quaternary amines. A representative chromatogram of an high salt ligand (ligand 6, 1,2-diaminoethane) is depicted in Fig. 2.

The results from anion-exchangers based on ligands composed of both primary and secondary amines indicate that the distance between the amine groups should be three CH_2 groups or less. This can be illustrated by comparing the breakthrough capacities of the anion-exchange media based on the ligands hydrazine (ligand 24), 1,2-diaminoethane (ligand 6), 1,3-diaminepropane (ligand 12), 1,4-diaminobutane (ligand 20) or 1,5-diaminopentane (ligand 19). Breakthrough capacities of anion-exchangers based on ligands 6 and 12 are about twice as high compared to the ligands 19, 20, and 24 (Table 2). However, the lower breakthrough value of the anion-exchanger based on hydrazine can probably be rationalized from the lower ligand density compared to the other ligands (Table 2). It is important to understand that the ligand density influences the chromatographic results to a great extent [17]. Table 2 shows that the Q_b-value of BSA and the elution conductivity of the three test proteins increase with ligand density. For example, three different degrees of tris(hydroxymethyl) aminomethane (0.21, 0.17 and 0.13 mmol/ml) resulted in Q_b-values of 20.3, 17.7 and 8.6 mg BSA/ml, respectively. The maximum binding capacity seems to level out at higher ligand densities. Similar relationships have been observed earlier [18].

For multi-modal anion-exchange media composed of only one type of secondary amine it seems beneficial for the Q_b-values of BSA if at least three hydroxyl groups are present close to the amine group. The best secondary anion-exchange medium is based on tris(hydroxymethyl) aminomethane (ligand 10). Compared to ligand 32 (2-amino-2-methyl-1,3-propanediol) which has only two hydroxyl groups but the same ligand density as ligand 10, the Q_b-value for

ligand 10 is about six times higher (Table 2). Furthermore, if the hydroxyl groups are situated three CH_2 groups from the amine (ligand 43) the Q_b-value is nearly 20 times lower compared to ligand 10. It can also be noted that if ethanolamine (ligand 26) is coupled to epoxy-activated Sepharose 6 Fast Flow with the same ligand density as for ligand 10 the Q_b-value of BSA will be four times lower (Table 2). However, if the ligand density of ethanolamine is increased to 0.36 mmol/ml (ligand 11) the breakthrough capacity approach the capacity obtained for the anion-exchanger based on ligand 10 (ligand density 0.21 mmol/ml).

Table 3
Recovery of BSA for some of the most promising high salt anion-exchange ligands

Ligand name (immobilised ligand code)	Recovery (%)
1,3-Diamino-2-propanol (1)	78
tris(2-Aminoethyl) amine (2)	90
1-Amino-4-guanidobutane (3)	94
1,3-Diamino-2-propanol (5)	91
1,2-Diaminoethane (6)	95
bis(3-Aminopropyl) amine (7)	87
1,3-Diaminopropane (8)	93
tris(Hydroxymethyl) aminomethane (10)	88
Pentaethylenhexamine (15)	83

The amount of BSA applied corresponds to 30% of the breakthrough capacity of BSA (see Section 2 for details).

3.2. Recovery of BSA

There is a range of important chromatographic properties that an ion-exchange medium aimed for large-scale protein purification must have to be an ideal packing material [19,20]. One of these important functional properties is the recovery of captured proteins. This is especially important for these types of anion-exchanger aimed for use at high salt conditions (28 mS/cm). Normally, desorption of samples from an ion-exchanger is performed with a salt or a pH gradient. For the high salt anion-exchangers the non-electrostatic interaction mechanisms must not be too strong preventing elution with salt. According to Table 3, recovery results show that all the better non-aromatic anion-exchangers (see Fig. 3 for ligand structures) have high recoveries of BSA (recoveries >78%) when eluted with salt (see Section 2). This proves that no secondary cooperative interactions are strong enough to retain the BSA when the electrostatic interactions have been eliminated. A breakthrough curve and an elution curve of BSA of the anion-exchanger based on ligand 4 (1,2-diaminoethane) are depicted in Fig. 4. The chromatographic conditions in this study have not been optimized, and therefore it should be possible to further improve the recoveries, for example by changing the pH of the desorption buffer (see below).

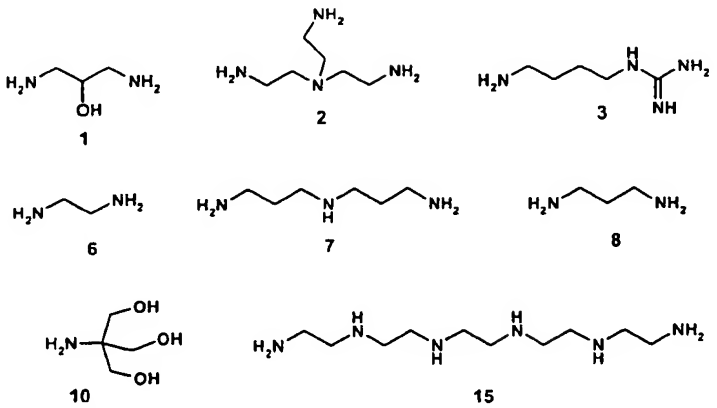


Fig. 3. Structures of non-aromatic anion-exchange ligands presented in Table 3.

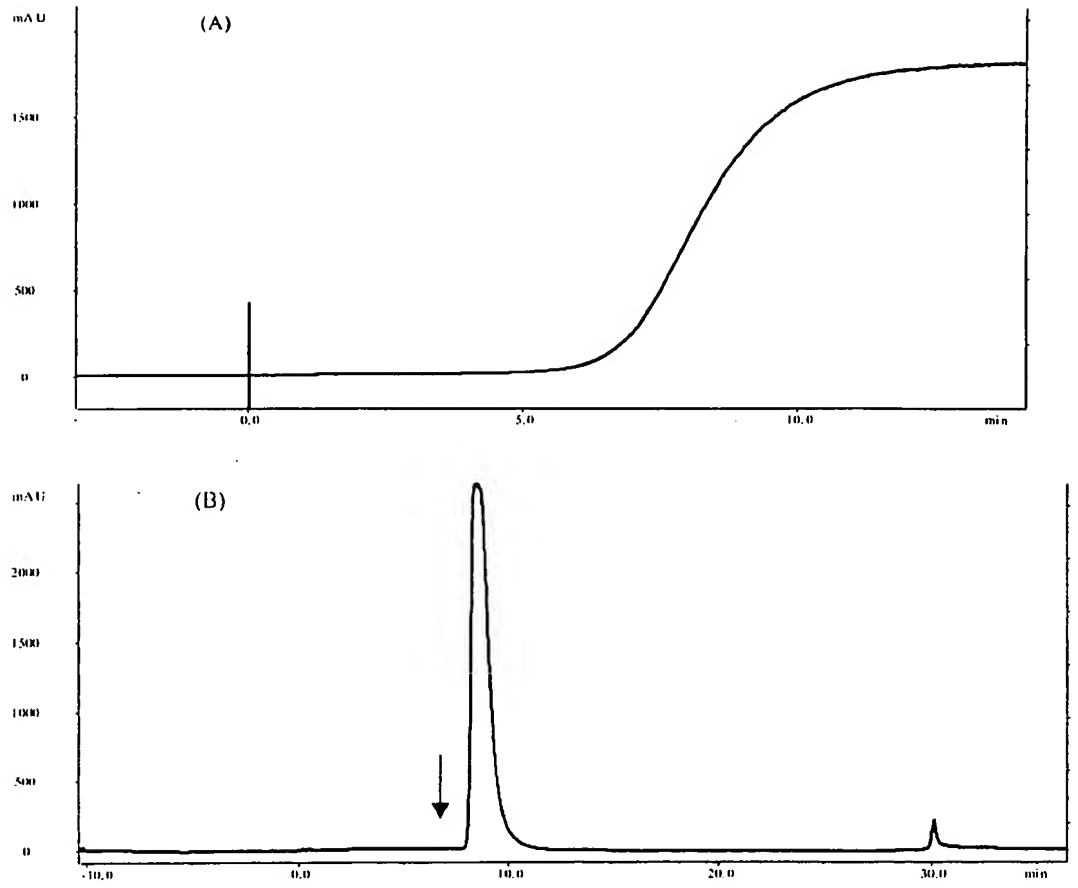


Fig. 4. Breakthrough curve of BSA for the anion-exchanger based on ligand 4 (1,2-diaminoethane) at high salt conditions (A). UV-profile of the elution curve for the recovery of BSA (B). The arrow in (B) indicates where the desorption buffer was applied. See Section 2 for more details.

3.3. Breakthrough capacity of BSA at low ionic-strength

It is well known that anion-exchangers based on quaternary and tertiary amines have high capacities of proteins when the sample is dissolved in buffers with low salt content (analytical concentration of the buffer corresponding to about 20 mM) [21,22]. Therefore, it is interesting to compare the breakthrough capacity of these new multi-modal weak anion-exchangers with a strong anion-exchanger at conditions where no extra salt has been added to the adsorption buffer. According to Fig. 5 Q Sepharose™ Fast Flow resulted in a breakthrough capacity of 69 mg of BSA/ml when a 20 mM Piperazine buffer (pH 6.0) was used and the best multi-modal anion-exchanger (based on ligands 4 or 5) resulted in a capacity of 54 mg/ml. This corresponds to ca 80% of the capacity of Q Sepharose™ Fast Flow that indicates that the new multi-modal anion-exchangers can efficiently capture proteins from low conductivity mobile phases. It can also be noted that the breakthrough capacity of BSA decreased from 69 to 1 mg/ml for Q Sepharose™ Fast Flow when 0.25 m NaCl was added to the adsorp-

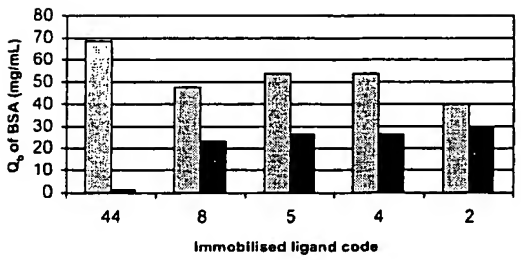


Fig. 5. Breakthrough capacity of BSA for Q Sepharose™ Fast Flow (ligand 44) and four new anion-exchangers at high salt conditions (black bars) and low salt conditions (grey bars). See Section 2 and Table 1 for ligand structures.

Table 4
The change of breakthrough capacity of BSA after 10 days incubation in 1 M sodium hydroxide solution of different non-aromatic anion-exchangers

Ligand name (immobilised ligand code)	Change[a] in Q_b-value of BSA (%)
tris(2-Aminoethyl) amine (2)	−3.9
1,3-Diamino-2-propanol (5)	−1.4
bis(3-Aminopropyl) amine (7)	0.5
1,3-Diaminopropane (8)	4.3
tris(Hydroxymethyl) aminomethane (10)	0.8

[a] The change in Q_b-value of BSA was calculated as: $(Q_b - Q_b^{NaOH})100/Q_b$ where Q_b and Q_b^{NaOH} are the breakthrough capacities of BSA before and after incubation, respectively.

tion buffer (20 mM Piperazine, pH 6.0). This change in mobile phase resulted only in a 50% decrease of breakthrough capacity when the new multi-mode anion-exchangers were used (Fig. 5).

3.4. Functional stability after treatment in 1.0 M sodium hydroxide solution

Ion-exchangers that are compatible with sodium hydroxide solutions are most suitable for large-scale production of biomolecules. Cleaning conditions in the production of pure biological materials require effective procedures of removing contaminating materials such as fatty acids, endotoxins and macromolecular aggregates. Furthermore, inactivation of living organisms, such as bacteria, yeasts and viruses within the chromatographic packing is of the utmost importance. Treatment of used media with 1 M NaOH solution can in many cases solve these contamination problems, therefore, it is desirable that anion-exchangers can sustain treatment with high pH solutions for extended time without decline in chromatographic performance [23]. To test the functional stability at alkaline conditions a number of multi-modal anion-exchangers were incubated for 1 week in 1.0 M NaOH solution. Table 4 shows that, according to the breakthrough results, the investigated anion-exchangers were not significantly influenced. These results are supported by an earlier investigation that showed that the anion-exchanger DEAE Sepharose™ Fast Flow can withstand harsh treatment at both high and low pH conditions (pH 1 and 14) [24].

3.5. Characterization of mixed-mode aromatic anion-exchangers aimed for capture of proteins at high salt conditions

A number of different amines containing some aromatic groups were tested as anion-exchange ligands. In Table 5 some of the breakthrough results are presented and in Fig. 6 is the ligand structures depicted. It can be noted that the most successful aromatic amines resulted in higher breakthrough capacities compared to the best non-aromatic anion-exchangers. However, it was not possible to desorb the captured proteins with high recovery by adding salt (2 M NaCl) to the mobile phase (Table 5). Furthermore, the function test showed that Lactalbumin and Soybean trypsin inhibitor could not be eluted with a linear salt gradient. In addition, it was not possible to desorb with a pH-step from pH 6–4. At pH 4 BSA and the ligand are positively charged. This indicates that secondary interactions [25] (probably hydrophobic interactions) or interactions between local negatively charged areas of BSA and the ligand are stronger than the electrostatic repulsion between the positively netto charged BSA and the ligand at pH 4. It can also be noted that BSA adsorbed to non-aromatic multi-modal anion-exchangers could easily be eluted with a pH-step to pH 4.0 with high recoveries. For example, anion-exchangers based on ligand 4 (1,2-diaminoethane) gave a recovery of BSA of 91%. These results clearly proved that the aromatic anion-exchangers have too strong secondary interactions to be practically useful. However, it has been shown that Benzamidine Sepharose™ Fast Flow can

Table 5
Ligand density, breakthrough capacity of BSA and the recovery of BSA after addition of an amount representing 30% of the breakthrough capacity and elution with B-buffer (20 mM Piperazine + 0.25 M NaCl, pH 6.0) for aromatic anion-exchange ligands

Immobilised ligand (Code)	Ligand density (mmol/ml)	Breakthrough capacity (mg/ml)	Recovery of BSA (%)
Thiocamine (48)	0.13	42.5	ne[a]
Tyrosinol (49)	0.13	39.1	ne
Tryptophanol (50)	0.15	37.4	37
Octopamine (51)	0.10	37.2	ne
2-Aminobenzimidazole (52)	0.17	33.6	82
2-Amino-3-phenylpropanol (53)	0.17	30.5	ne
1-Aminoethyl-4-hydroxybenzyl alcohol (54)	0.09	29.2	49
2-(4-Aminophenyl) ethylamine (55)	0.20	26.0	ne
Noradrenaline (56)	0.08	25.2	ne
2-Amino-1-(4-nitrophenyl)-1,3-propanediol (57)	0.09	23.3	ne

[a] ne: Not eluted.

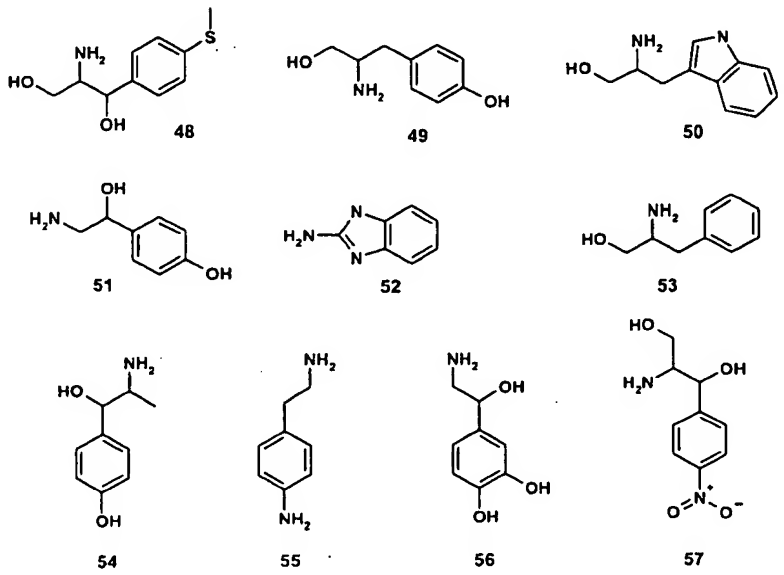


Fig. 6. Structures of aromatic anion-exchange ligands presented in Table 5.

work properly as an anion-exchanger due to its extremely low ligand density (0.02 mmol/ml) [26]. In the specific case of phenol containing ligands such as tyrosinol or Octopamine, desorption of BSA can be achieved by a pH-step to pH 9.0. Under these conditions the phenol group is partially negatively charged. This facilitates desorption of the negatively charged BSA by repulsion and good recoveries (larger than 90%) can therefore be obtained. Unfortunately, these phenolic ligands have a low stability under normal cleaning in place procedures and would only be of interest for very particular applications.

4. Conclusion

Multi-modal anion-exchangers have been selected to capture proteins from solutions of high conductivity (28 mS/cm). One of the most distinctive features of these multi-modal anion-exchangers is that the lig-

ands are based on non-aromatic amines. The optimal ligands are constructed from primary and secondary amines or based on both types of these amines. In all cases the presence of hydrogen donor groups in the proximity of the anion-exchanger group, seems to be essential for the ability of the ligand to function in high salt milieu. The captured proteins can then easily be desorbed with high recovery by increasing the ionic strength or by a pH-step to conditions where both proteins and ligands are positively charged. Multi-modal non-aromatic anion-exchangers will provide additional flexibility in the design of new purification processes, and the possibility to capture proteins directly from different feedstock will be investigated in the near future. It should also be pointed out that these new ligands are weak ion-exchange ligands (primary and secondary amines) and therefore can not be used at pH-values higher than about 9.

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